Reversible Adsorption and Nonreversible Insertion of *Escherichia coli* α-Hemolysin into Lipid Bilayers

Laura Bakás,* Helena Ostolaza,* Winchil L. C. Vaz,# and Félix M. Goñi*
*Grupo Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, 48080 Bilbao, Spain, and #Unidade de Ciências Exactas e Humanas, Universidade do Algarve, P-8000 Faro, Portugal

ABSTRACT α-Hemolysin is an extracellular protein toxin (107 kDa) produced by some pathogenic strains of *Escherichia coli*. Although stable in aqueous medium, it can bind to lipid bilayers and produce membrane disruption in model and cell membranes. Previous studies had shown that toxin binding to the bilayer did not always lead to membrane lysis. In this paper, we find that α-hemolysin may bind the membranes in at least two ways, a reversible adsorption and an irreversible insertion. Reversibility is detected by the ability of liposome-bound toxin to induce hemolysis of added horse erythrocytes; insertion is accompanied by an increase in the protein intrinsic fluorescence. Toxin insertion does not necessarily lead to membrane lysis. Studies of α-hemolysin insertion into bilayers formed from a variety of single phospholipids, or binary mixtures of phospholipids, or of phospholipid and cholesterol, reveal that irreversible insertion is favored by fluid over gel states, by low over high cholesterol concentrations, by disordered liquid phases over gel or ordered liquid phases, and by gel over ordered liquid phases. These results are relevant to the mechanism of action of α-hemolysin and provide new insights into the membrane insertion of large proteins.

INTRODUCTION

Escherichia coli α-hemolysin (HlyA) is a 107-kDa protein secreted by pathogenic strains of this bacterium and belongs to the so-called RTX protein family, characterized by a glycine-rich nonapeptide repeat region near the C-terminal end (see Coote, 1992, for a review). In addition to this repeat domain, the protein has a hydrophobic region near the N-end (Menestrina et al., 1994, 1995), and a C-terminal signal peptide (Zhang et al., 1995; Chervaux and Holland, 1996). The mature protein contains two fatty acyl residues linked to internal lysines (Stanley et al., 1994). HlyA disrupts eukaryotic cell membranes and forms cation-selective channels in planar lipid membranes (Menestrina et al., 1995). The toxin is also capable of inducing leakage of phospholipid large unilamellar vesicles (Ostolaza et al., 1993). In general, there is ample experimental evidence that HlyA, although existing in soluble form after its secretion, may become membrane-associated to produce membrane disruption.

The mechanism of HlyA insertion in lipid bilayers is not known in detail. Besides the fact that some cells might contain HlyA receptors, pure lipid bilayers and vesicles constitute a good model for this study. We have recently described methods for separately measuring toxin binding to membranes and toxin-induced lysis, and found that binding is not necessarily followed by membrane damage. In particular, HlyA binds lipid bilayers with about the same affinity in the presence or absence of Ca^{2+}, but only when the protein has been preincubated with this cation does the lytic effect follow toxin binding (Ostolaza and Goñi, 1995). In this paper, we explore in more detail the influence of some lipid properties on HlyA binding to membranes. The interest of this study goes beyond the mechanism of action of the toxin, because it is one case of the more general, and important, biological problem of protein insertion in membranes (see reviews by Jain and Zakim, 1987; Hannavy et al., 1993; Isenman et al., 1995; see also, to mention but a few examples, Dibble et al., 1993; Sankaram et al., 1994; Pott and Dufourc, 1995; Rytömaa and Kinnunen, 1995).

The present study is specifically directed to exploring the influence of lipid phases on the binding of HlyA. Particular attention is paid to the case of coexisting fluid phases, such as phospholipid-cholesterol fluid ordered and fluid disordered phases (Sankaram and Thompson, 1991; Almeida et al., 1992; Monette et al., 1993; Mateo et al., 1995; McMullen and McElhaney, 1995; Pott and Dufourc, 1995), and to the case of coexisting gel and fluid phases (Vaz et al., 1989; Jorgensen et al., 1993; Sankaram et al., 1992, 1994; Piknová et al., 1996). Tomita et al. (1992) have studied the influence of membrane fluidity on the assembly of *Staphylococcus aureus* α-toxin, a channel-forming protein not belonging to the RTX family, in liposomal membranes. Following a similar experimental reasoning, we have established bilayer conditions under which HlyA may bind lipid membranes in either a reversible or an irreversible way.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) was grade I from Lipid Products (South Nutfield, England). Dioleoyl, dimyristoyl, dipalmitoyl, and distearoylphos-

Received for publication 2 May 1996 and in final form 3 July 1996.

Address reprint requests to Dr. Felix M. Goñi, Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain. Tel.: 34-4-464-7700, ext. 2407; Fax: +34-4-464-8500; E-mail: gbzoseth@lg.ehu.es.

Dr. Bakás's permanent address is Cátedra de Biología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina.

0006-3495/96/10/1869/08 $2.00

phatidylcholine (respectively, DOPC, DMPC, DPPC, and DSPC) were supplied by Avanti Polar Lipids (Alabaster, AL). Cholesterol (Ch) was from Sigma. 1-Amino-naphthalene-1,3,6-trisulfonate (ANTS) and *p*-xylenebispyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR). Plasmid-encoded α-hemolysin (HlyA) was purified from the culture filtrates of an overproducing strain of *E. coli*, according to the method of Ostolaza et al. (1991); before its use, the protein was dialyzed against 150 mM NaCl, 6 M urea, 20 mM Tris/HCl, pH 7.0 (TCU buffer), to which 1 mM EGTA was added. Horse red blood cells were supplied by Microlab (Madrid, Spain).

Large unilamellar vesicles (LUVs) of different compositions were prepared by extrusion and sized using 0.1-μm pore size Nuclepore membranes as described by Mayer et al. (1986); the buffer was 20 mM Tris-HCl, 150 mM NaCl, pH 7.0 (TC buffer), ± EGTA, Ca^{2+}, or Zn^{2+} as required.

Measurements of intrinsic fluorescence of HlyA

Bilayer-toxin interactions were monitored through changes in the intrinsic fluorescence emission spectra of HlyA. Small aliquots of a concentrated LUV suspension were added to a protein solution (0.15–0.30 μM) in a cuvette with continuous stirring. After equilibrating for 5 min, emission spectra were recorded with an excitation light of 295 nm (slit 5 mm). Fluorescence intensity measurements were corrected for light scattering (Surewicz and Epand, 1984).

Toxin binding to liposomes and toxin transfer to erythrocytes

The method is essentially the one described by Tomita et al. (1992). Horse red blood cells were used as indicators in assessing the hemolytic activity of HlyA bound to multilamellar vesicles (MLVs). Toxin (20–30 μg) and liposomes (multilamellar vesicles, 1 mM) were incubated in TC buffer with 10 mM $CaCl_2$ for 30 min, at the required temperature. HlyA-liposome complexes were recovered by centrifugation (16,000 × *g*, 15 min, 4°C) and washed three times to remove any unbound toxin. The complexes were resuspended in 200 μl of cold buffer and used for assays of hemolytic activity, as well as in protein and lipid determination. This was called the "centrifugation method" for measuring total protein bound to liposomes.

For hemolytic activity assays, an aliquot of the HlyA-liposome complexes was serially diluted with cold buffer in a 96-well microtiter plate. One hundred microliters of the dilute suspensions were mixed with 100 μl of a standardized suspension of red blood cells and left to incubate for 30 min at 37°C. The absorbance of supernatants was read at 412 nm. One hundred percent lysis was established after lysing the red blood cells with Triton X-100 (1% final concentration).

Time-resolved experiments and equilibrium conditions

Preliminary time-resolved experiments using the various techniques described above showed that 5 min after toxin addition, the various cell and model systems used in this study had reached equilibrium. This is what would be expected from a diffusion-limited process, as is probably the case with toxin binding and insertion. Thus all measurements have been carried out 5 min after HlyA addition, except in those cases in which, for the sake of convenience, equilibration was allowed to take place for 30 min, as stated in each case.

RESULTS

The capacity of liposome-bound HlyA to produce red blood cell lysis was tested after incubating the protein with DOPC, DMPC, or DPPC multilamellar liposomes at 0°C and 37°C. The results are presented in Fig. 1. The dotted line corresponds to the dose-response curve of free hemolysin, i.e., protein that has been preincubated in the absence of liposomes and then tested against red blood cells. When HlyA has been preincubated with DMPC liposomes at 0°C (i.e., well below T_c, the main gel-to-fluid transition temperature of the phospholipid; open circles), incubation with horse erythrocytes leads to hemolysis, although not all of the

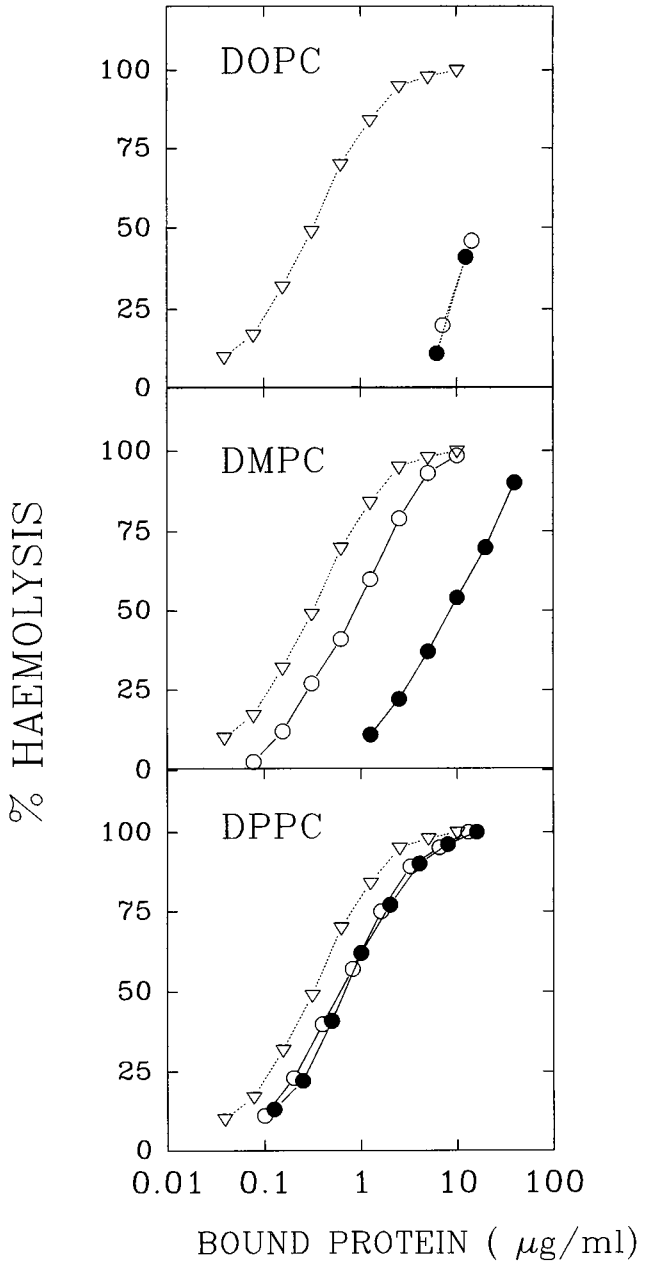

FIGURE 1 Accessibility of liposome-bound α-hemolysin to red blood cells. HlyA was incubated for 30 min at 0°C (○) or 37°C (●) with multilamellar liposomes of DOPC, DMPC, or DPPC, as indicated. The resulting liposome-protein complexes were further incubated with horse erythrocytes, and the hemolytic activity was recorded. See Materials and Methods for details. Dotted line: hemolytic activity of native toxin, i.e., preincubated in the absence of liposomes and then added to the erythrocyte suspension.

protein seems to be capable of producing cell lysis, because the dose-response curve is somewhat shifted to higher protein concentrations. However, when preincubation with DMPC (T_c = 23°C) has taken place at 37°C (filled circles), the fraction of HlyA available for hemolysis is much smaller, and the dose-response curve is shifted by about an order of magnitude. This can be quantitatively stated by comparing the amount of protein required in all three cases to produce 50% hemolysis. The data are shown in Table 1, and the protein concentrations are, respectively, 0.5, 0.95, and 10.0 μg/ml for HlyA preincubated without and with liposomes at 0°C and at 37°C. In terms of percentage, 53% of the protein preincubated at 0°C but only 5% of HlyA preincubated at 37°C with DMPC is "available" for hemolysis. The phenomenon appears to be related to the physical state of the bilayer, because HlyA preincubated with multilamellar vesicles of DPPC (T_c = 41.5°C) remains available for hemolysis when incubated at 0° or 37°C (Fig. 1 and Table 1). The opposite situation is found with DOPC, which remains fluid at both temperatures; the proportion of HlyA available for hemolysis is very small in both cases, and values producing 50% hemolysis (obtainable only by extrapolation) are virtually similar and comparable to the data for preincubation with DMPC in the fluid state (37°C). Note that protein binding to the multilamellar liposomes is essentially similar in all cases (Table 1), irrespective of temperature or lipid composition. Control experiments in which protein-free liposomes are incubated with red blood cells showed that none of the vesicle preparations had any hemolytic character at all.

These results are interpreted in terms of the existence of two populations of membrane-bound HlyA molecules, respectively, reversibly and irreversibly bound. Lipid bilayers in the fluid state favor irreversible binding. Our previous studies with LUV in the fluid state indicated that, under conditions leading to membrane leakage, the toxin remained irreversibly bound to the membrane (Ostolaza et al., 1993). In the case of fluid MLV (e.g., DOPC, or DMPC at 37°C), virtually all of the toxin (>90%) remains irreversibly bound (Table 1). For bilayers in the gel state (e.g., DPPC, or DMPC at 0°C), the apparent proportion of reversibly bound HlyA is far from 100%; this result is observed either because some of the protein is irreversibly bound even below T_c, or because not all of the reversibly bound hemolysin binds red blood cells under conditions leading to leakage (Ostolaza and Goñi, 1995). A similar situation below T_c was found for *S. aureus* α-toxin (Tomita et al., 1992).

Some of the former experiments with DMPC multilamellar vesicles were repeated with preincubated HlyA and liposomes in TC buffer but with 1 mM $ZnCl_2$, or 1 mM EGTA, instead of Ca^{2+}. These experiments were performed because previous studies had shown that in the absence of divalent cations, or in the presence of Zn^{2+}, HlyA was inactive (Ostolaza et al., 1995). In agreement with the previous observations, no hemolysis was detected under those conditions, in spite of the fact that red blood cells were suspended in a calcium-containing buffer (data not shown). This suggests that, in the transfer from MLV to erythrocyte (whenever this occurs), the calcium-binding domain of HlyA (Ostolaza et al., 1995) does not unfold to allow entry of calcium ions and subsequent protein activation.

The hypothesis that HlyA becomes irreversibly bound to lipid bilayers when they are in the fluid state is also supported by an experiment in which toxin binding is assessed as an increase in the intrinsic Trp fluorescence of the protein (Surewicz and Epand, 1984; Ostolaza and Goñi, 1995). Fig. 2 shows the relative change in HlyA intrinsic fluorescence when incubated continuously with increasing temperatures in the presence of DMPC LUVs (T_c = 23°C). Fluorescence increases steeply between 20°C and 23°C, i.e., near the gel-fluid transition. If a system equilibrated well above T_c is then gradually cooled down (Fig. 2, triangles), the intrinsic fluorescence does not vary. Our interpretation of these data is that HlyA becomes inserted in the DMPC bilayer in a nonreversible way, particularly when the lipid is in the fluid phase. The tryptophanyl residues being in a less polar environment than before, the fluorescence emission intensity increases. Once the protein is inserted above T_c, cooling does not reverse the fluorescence effect, because binding above T_c is irreversible. The peptide becomes kinetically trapped in the gel state. Note that direct measurements of liposome-bound protein do not reveal major differences between the gel and the fluid phase (Table 1), probably because the direct centrifugation method gives an estimate

TABLE 1 Binding of α-hemolysin to phospholipid liposomes and transfer of α-hemolysin from liposome-toxin complexes to horse erythrocytes

	% Binding*		HlyA concentration (μg/ml)#		% Residual activity§	
Phospholipid	0°C	37°C	0°C	37°C	0°C	37°C
DOPC	10.9	15.3	15.5¶	16.9¶	3.2¶	3.0¶
DMPC	10.2	14.7	0.95	10.0	53	5.0
DPPC	9.8	13.0	0.78	0.81	64	61

*Total protein bound, according to the centrifugation method.

#Concentration of HlyA, in the form of toxin-liposome complex, that produces 50% hemolysis in the standard preparation of horse red blood cells. The corresponding value for free HlyA is 0.5 μg/ml.

§The percentage residual hemolytic activity in the toxin-liposome complexes is calculated as (concentration of native toxin required to produce 50% hemolysis/concentration of liposome-bound toxin required to produce 50% hemolysis) × 100 (Tomita et al., 1992).

¶Extrapolated values.

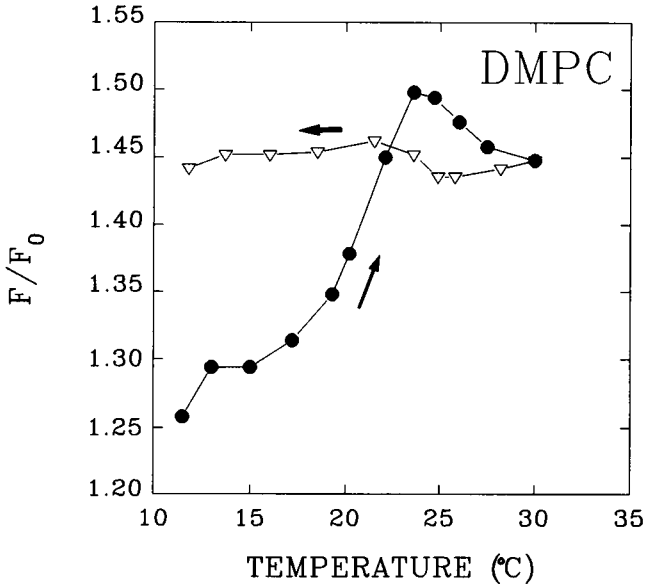

FIGURE 2 The influence of gel-fluid phase transition of DMPC on HlyA binding to LUVs, assessed as an increase in intrinsic Trp fluorescence of the protein. ●, Heating run; ▽, cooling run.

of total (reversibly + irreversibly) bound protein, whereas intrinsic fluorescence increases noticeably only when the protein becomes irreversibly embedded in a lipid bilayer.

Binary phospholipid mixtures offer various possibilities of phase transitions and of coexistence of gel and fluid phases. We have studied the incorporation of HlyA into various mixtures of DMPC and DSPC at 30°C. At this temperature, the binary lipid system is in the liquid crystalline phase for a DSPC molar fraction $x \leq 0.15$, and in the gel phase for $x \geq 0.65$ (Knoll et al., 1981; Vaz et al., 1989; Sankaram et al., 1992; Jorgensen et al., 1993; Piknová et al., 1996); between these boundaries, the gel and liquid crystalline phases coexist. Total protein bound, according to the centrifugation method, was $3 \pm 0.1/10^4$ (protein/lipid mol ratio) at 30°C, irrespective of the system composition. HlyA irreversible binding to DMPC/DSPC LUVs is shown in Fig. 3 *A*, as detected through changes in the intrinsic fluorescence. Again insertion appears to be at a maximum when the system is in the fluid phase, lower when both phases coexist, and virtually zero when the lipids are in the gel phase. A small but reproducible increase observed between $x_{DSPC} = 0.00$ and $x_{DSPC} = 0.05$ is attributed to a facilitated protein insertion when mixed-length phospholipid chains coexist. The proportion of inserted protein does not decrease precisely in parallel with the theoretical fraction of fluid phase (Fig. 3 *A*, dotted line). This may be due to fluctuations in the phase boundaries induced by the presence of the protein. Our differential scanning calorimetry studies of phospholipid-HlyA systems indeed show a protein-dependent widening of the main lipid gel-fluid phase transition (P. Veiga et al., unpublished data). Insertion of a small (25 aa) peptide is known to leave the phase boundaries unmodified (Sankaram et al., 1994), but the behavior of the much larger

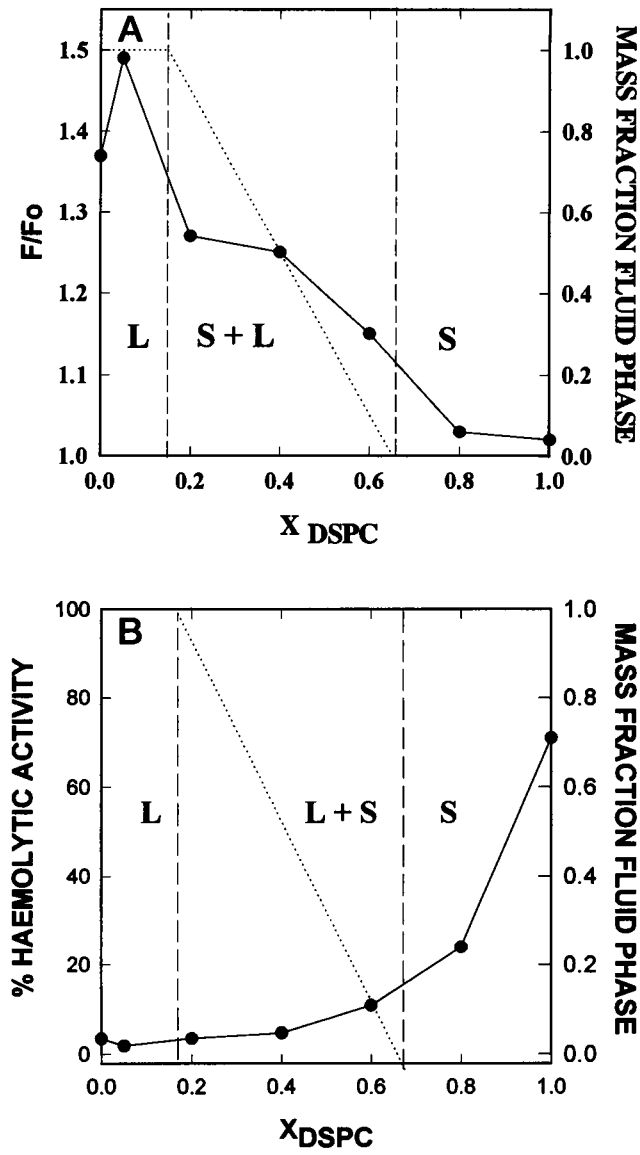

FIGURE 3 Binding of α-hemolysin to bilayers composed of binary phospholipid mixtures DMPC/DSPC. (*A*) Irreversible LUV binding measured as an increase in intrinsic Trp fluorescence of the protein. Data relative to fluorescence intensity of the pure protein in buffer, in the absence of lipids. (*B*) Accessibility of MLV-bound HlyA to red blood cells (as in Fig. 1). Measurements were carried out at 30°C. The two discontinuous vertical lines indicate the pure lipid phase boundaries. The dotted line corresponds to the calculated mass fraction of fluid phase as a function of system composition.

and more complex HlyA may be different, particularly in view of the small size of the fluid phase domains in DMPC/DSPC mixtures at 30°C (Piknová et al., 1996). The putative effect of the protein on the vesicle curvature and subsequently on the phase behavior of the lipid mixture (Brumm et al., 1996) should also be taken into account in our system.

When the reversibility of HlyA binding to DMPC/DSPC bilayers in the form of MLVs is tested by incubating liposome-bound toxin with horse erythrocytes, the results are rather straightforward (Fig. 3 *B*). As long as there is some lipid in the

fluid phase, binding is essentially irreversible—no hemolysis is detected. Only when the mixture is in the gel phase, and particularly for $x_{DSPC} = 1.0$, does the binding become reversible. The point at $x_{DSPC} = 0.8$ is interesting because it shows virtually the same F/F_o as $x_{DSPC} = 1.0$ (Fig. 3 *A*), yet the recovery of hemolytic activity is much smaller in the former case. One plausible explanation is that $x_{DSPC} = 0.8$ represents a limiting situation; in fact, the results in Fig. 3 may suggest that, in the presence of HlyA, the lipid phase boundaries are in fact near $x_{DSPC} = 0.05$ and 0.8, respectively. Cholesterol/phosphatidylcholine mixtures are interesting because they offer the possibility of coexisting ordered and disordered liquid phases (Vist and Davis, 1990; Sankaram and Thompson, 1991; Almeida et al., 1992; Mateo et al., 1995; McMullen and McElhaney, 1995). For DMPC/cholesterol, the phase diagram published by Almeida et al. (1992) shows, at 10°C, a gel phase for 7 mol% cholesterol, an ordered liquid phase at 30 mol% cholesterol, and coexisting gel and ordered liquid phases between. At 37°C, the phases are disordered liquid, disordered liquid + ordered liquid, and ordered liquid, the two corresponding boundaries occurring at ~10mol% and ~30 mol% cholesterol. The total amount of liposome-bound HlyA estimated by the centrifugation method for bilayers composed of DMPC/cholesterol decreases steadily and slowly with increasing proportions of cholesterol (Fig. 4), apparently with little or no influence of temperature or phase transitions, as was the case for pure DMPC bilayers (Table 1).

When irreversible HlyA insertion into LUV bilayers is considered, assessed by the intrinsic fluorescence method (Figs. 5 *A* and 6 *A*), the general tendency is, as seen for total binding in Fig. 4, a decrease in irreversible binding as the cholesterol concentration increases. At 10°C (Fig. 5 *A*), the boundary between S and S + L_o does not mark any important change in toxin binding, but beyond the boundary between S + L_o and L_o binding is clearly decreased, suggesting that the liquid ordered state is not a favorable solvent for HlyA. At 37°C the bilayer ability to bind hemolysin decreases steeply as soon as some L_o phase is formed, suggesting again the low affinity of L_o for the toxin. Note that for cholesterol concentrations at or near 50%, when the DMPC gel-fluid transition has been completely smeared out, HlyA binding at 10°C and 37°C is virtually the same.

Tests of accessibility to red blood cells of HlyA bound to DMPC/cholesterol MLVs (Figs. 5 *B* and 6 *B*) confirm in general the previous observations. At 37°C, accessibility is at

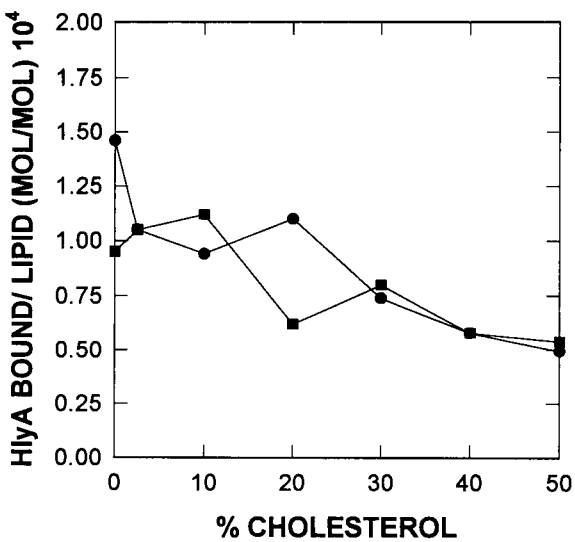

FIGURE 4 Total binding (measured by the centrifugation method) of HlyA to bilayers composed of DMPC:cholesterol mixtures. Measurements carried out at 10°C (■) and 37°C (●).

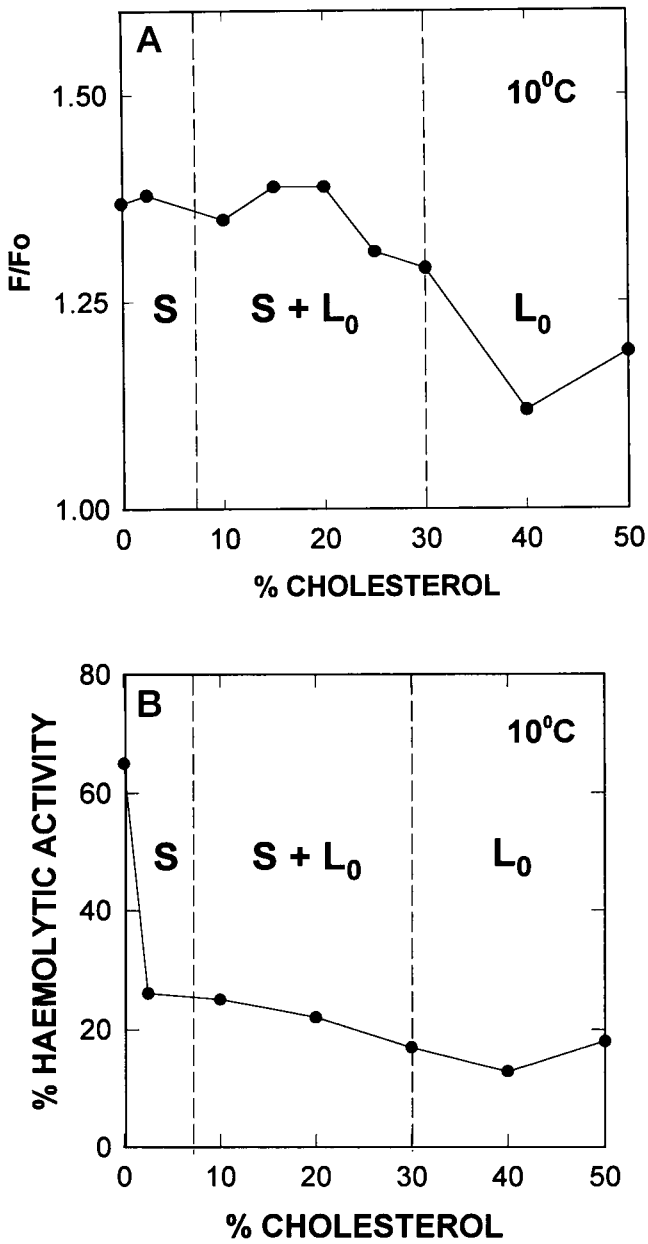

FIGURE 5 Binding of α-hemolysin to bilayers composed of DMPC/cholesterol at 10°C. (*A*) Irreversible LUV binding measured by the intrinsic fluorescence method. (*B*) Accessibility of MLV-bound toxin to erythrocytes (as in Fig. 1). The discontinuous vertical lines correspond to the lipid phase boundaries.

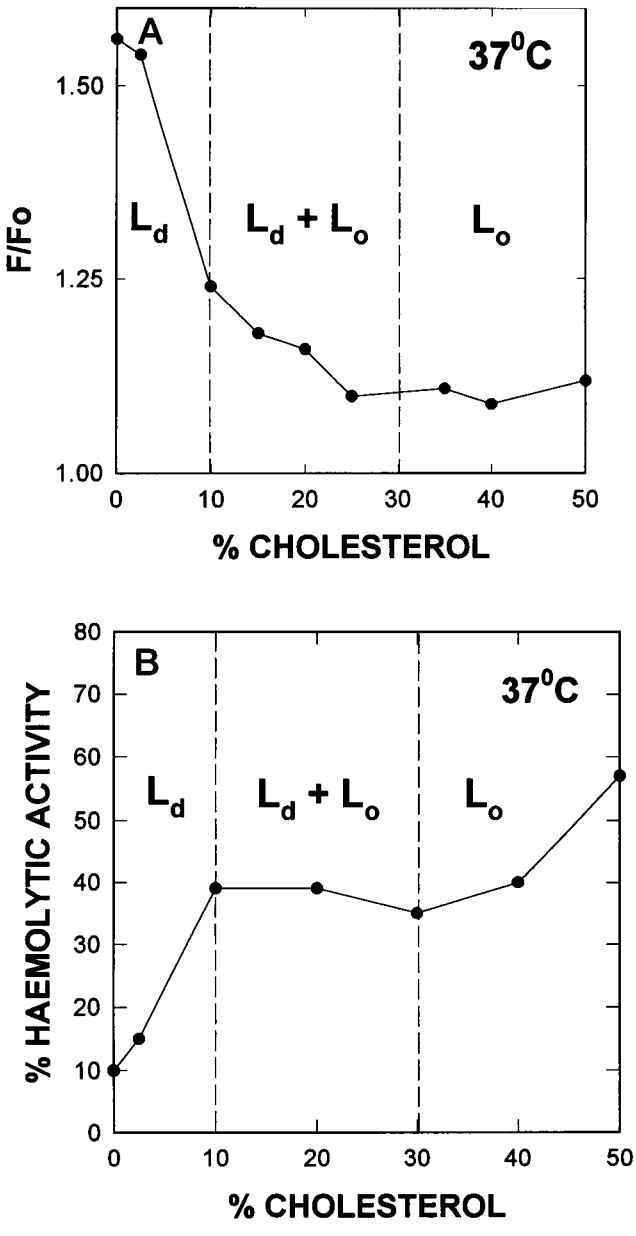

FIGURE 6 Binding of α-hemolysin to bilayers composed of DMPC/cholesterol at 37°C. (*A*) Irreversible LUV binding measured by the intrinsic fluorescence method. (*B*) Accessibility of MLV-bound toxin to erythrocytes (as in Fig. 1). The discontinuous vertical lines correspond to the lipid phase boundaries.

a minimum when the bilayer is in the L_d state and at a maximum when the L_o state predominates (Fig. 6 *B*). At 10°C, two remarkable features are observed (Fig. 5 *B*). One is the very high hemolytic activity for HlyA bound to pure DMPC at 10°C (in accordance with the data in Table 1), followed by a dramatic decrease as soon as 5% cholesterol is added. The 95:5 DMPC/cholesterol mixture should be in the gel state as much as the pure DMPC, but the large decrease in hemolytic activity (with a similar extent of binding according to the fluorescence measurements; Fig. 5 *A*) suggests again that the protein is changing the phase behavior of the mixture. The second noteworthy observation is that, at the temperature of 10°C, the liquid ordered phase is somehow stabilizing the membrane-bound form of the toxin—not only does the recovery of hemolytic activity decrease and remain low as soon as some cholesterol (and presumably some L_o phase) is present (Fig. 5 *B*), but the recovery from pure L_o (50% cholesterol) is also lower at 10°C than at 37°C (Fig. 6 *B*). This is an indication that, for a given phospholipid/cholesterol mixture in the same phase at two different temperatures, even when the proportion of irreversibly bound HlyA is similar (Figs. 5 *A* and 6 *A*), the fraction of protein transferred from liposomes to erythrocytes is lower at the lower temperature, perhaps indicating a temperature-dependent differential organization of the protein in the bilayer. In turn, this suggests that the mode of incorporation of HlyA to lipid bilayers may be more complex than the two possibilities (reversible/irreversible) mentioned above. With this limitation in mind, we can conclude that the lipid bilayer properties that facilitate the irreversible insertion of HlyA are the fluid state over the gel state (Figs. 1–3), the low over the high cholesterol concentrations (Figs. 5 and 6), the disordered liquid (L_d) over the ordered liquid (L_o) or gel states (Figs. 5 and 6), and the gel over the liquid ordered state (Figs. 5 and 6).

DISCUSSION

The mechanism of membrane lysis by α-hemolysin

Our previous studies on this protein have shown that 1) a specific receptor is not required for membrane lysis to occur, because leakage of large molecules is induced by HlyA in vesicles of defined lipid composition, e.g., pure egg PC (Ostolaza et al., 1993); 2) HlyA may bind lipid bilayers under conditions (e.g., absence of Ca^{2+}) that do not lead to lysis (Ostolaza and Goñi, 1995). The data in this paper provide us with a closer look at the binding step. The binding data obtained by the centrifugation method and by the intrinsic fluorescence method do not always agree (e.g., Table 1 and Fig. 2), particularly because the fluorescence procedure shows differences between binding to the gel and fluid phases, whereas the centrifugation method does not. This had not been detected in former studies because only bilayers in the fluid phase were tested. When the binding data are complemented with assays of accessibility of liposome-bound HlyA to erythrocytes, two populations can be distinguished in the membrane-bound toxin fraction, one that is transferred to the red blood cells (reversibly bound HlyA) and one that is not (irreversibly bound). The fact that irreversible but not reversible binding is characterized by changes in intrinsic fluorescence supports the hypothesis that "binding" actually includes two distinct phenomena, reversible adsorption of the protein to the bilayer and irreversible insertion into the same. Adsorption would be an early step that, in certain cases (e.g., fluid bilayers), would be followed by insertion. Adsorption appears to be largely independent of bilayer fluidity or composition (at least in the absence of net surface charge), whereas insertion is highly dependent on the bilayer physical properties (see Results).

By combining data from this and previous papers (Ostolaza et al., 1995; Ostolaza and Goñi, 1995), it can be concluded that irreversible insertion does not lead automatically to membrane lysis (see the case of binding in the presence of EGTA, accompanied by a similar increase in fluorescence than in the presence of Ca^{2+}). A further phenomenon is required that occurs only in the presence of Ca^{2+}. In α-toxin of *S. aureus* (Tomita et al., 1992), as well as in certain pore-forming cytolysins (Bhakdi and Tranum-Jensen, 1986), insertion is followed by oligomerization. However, in the case of HlyA, no oligomer has been isolated up to now, although circumstantial evidence in its favor has been produced (Ostolaza et al., 1993; Ludwig et al., 1993). No data are currently available on the step(s) between HlyA insertion and membrane disruption.

The insertion of α-hemolysin in lipid bilayers

A fraction of the membrane-associated α-hemolysin is irreversibly bound to the bilayer, its Trp residues appear to be in a less polar environment than when the protein is free in solution, and, under the appropriate conditions (e.g., presence of Ca^{2+}), it causes bilayer leakage and/or disruption. These experimental facts indicate that this particular fraction has become inserted in the bilayer. Apart from the already discussed implications for the mechanism of toxin action, the experimental data may be of interest in the framework of peptide insertion in membranes, the first stage in membrane protein folding (Lemmon and Engelman, 1994).

Unlike the cases of protein translocation in prokaryotes (de Kruijff, 1994), anionic phospholipids are not required for HlyA insertion (Ostolaza et al., 1993). Jain and Zakim (1987) have pointed out the requirement that hydrophobic regions of the bilayer become transiently exposed to the aqueous phase for protein incorporation to occur. Such transient exposure would be favored by overall changes in the bilayer properties, such as fluidity (Chapman, 1975), or by particular properties of a localized microenvironment, or defects (Jain and Zakim, 1987). The requirement of fluid bilayers for insertion is shown in Fig. 1, and more explicitly in Fig. 3, where, even if gel and fluid phases may coexist, the proportion of irreversibly bound protein decreases with the fraction of fluid phase. This is in agreement with the preferential partitioning of gramicidin A′ in fluid phospholipid phases coexisting with gel phases (Dibble et al., 1993). The uneven distribution of a transmembrane peptide into different coexisting lipid domains has also been described by Zhang et al. (1995). Moreover, Polozov et al. (1995) described the insertion of two amphipathic peptides in zwitterionic phospholipid bilayers in the fluid, but not in the gel state; thus the preference for fluid bilayers by the peptides to be inserted appears to be a rather general and predictable phenomenon.

Defects and intrinsic instabilities in the bilayer may promote protein insertion because defect sites may accommodate a protein molecule without inducing additional energetically unfavorable general disorder, desolvation, or lateral compression (Jain and Zakim, 1987). Local defects may arise from lipid mixtures (McIntosh et al., 1983), or from the presence of impurities (e.g., detergents) or other proteins, etc. Our experimental results show that conditions under which local defects are likely to occur (e.g., disordered liquid state; Fig. 6) do favor irreversible protein insertion. The overall effect of cholesterol is to make protein insertion more difficult, probably because its rigid structure does not help to accommodate the rough protein surface (Figs. 5 *A* and 6 *A*). Note also that cholesterol tends to increase bilayer thickness (Levine and Wilkins, 1971; Nezil and Bloom, 1992) and integral proteins tend to partition into domains of a given thickness (Bretscher and Munro, 1993). The properties of cholesterol are evident in the cholesterol-rich ordered liquid phases (Figs. 5 *A* and 6 *A*), which support very little insertion. Almeida et al. (1992) have explained this phenomenon in terms of cholesterol occupying free volume in the bilayer. It is interesting, in this respect, that *S. aureus* α-toxin hexamerization is favored by these cholesterol-rich phases (Tomita et al., 1992). However, the relationship between the presence of cholesterol and the reversibility of HlyA binding appears to be more complex than the general anti-insertion effect just discussed. The effects in the DMPC/cholesterol mixture at 10°C are a good example, particularly the surprisingly low recoveries of hemolytic activity in the presence of cholesterol (Fig. 5 *B*) that do not correlate with the binding data (Fig. 5 *A*). Moreover, the irreversible binding data are very similar for the L_o phase at 10°C and 37°C (Figs. 5 *A* and 6 *A*), whereas the recovery of hemolytic activity is much higher at the higher temperature (Figs. 5 *B* and 6 *B*). Bretscher and Munro (1993) have proposed that changes in cholesterol concentration in cell membranes may induce segregation of domains with high and low cholesterol concentrations; this idea has received support from the biophysical studies of Virtanen et al. (1995) in model membranes. If this were the case, segregation of domains would be favored by low temperatures, and HlyA would partition into domains of a particular composition, irrespective of the average cholesterol contents in the mixture.

The fact that protein insertion itself helps to promote new structural defects is important because it would support a cooperative process of insertion, which in turn is related to the putative oligomerization of HlyA (Ostolaza et al., 1993; Ludwig et al., 1993). Pott and Dufourc (1995) have underlined the creation of defect structures in the membrane upon the addition of melittin that might modify the overall elastic properties of the membrane, and Monette et al. (1993) proposed that high cholesterol concentrations induce tight lipid packing, which in turn prevents penetration of melittin into the bilayer. In our system, even the gel state is more prone to HlyA insertion than the L_o one, probably because of the structural defects that are known to exist in liposomes below T_c.

This work was supported in part by DGICYT grant PB91/0441 and by a Human Capital and Mobility Network from the European Union.

REFERENCES

Almeida, P. F. F., W. L. C. Vaz, and T. E. Thompson. 1992. Lateral diffusion in the liquid phases of dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry.* 31: 6739–6747.

Bhakdi, S., and J. Tranum-Jensen. 1986. Membrane damage by channel-forming proteins. Staphyloccal alpha-toxin, streptolysin-O and the C5B-9 complement complex. *J. Immunol.* 136:2999–3005.

Bretscher, M. S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science.* 261:1280–1281.

Brumm, T., K. Jorgensen, O. G. Mouritsen, and T. M. Bayerl. 1996. The effect of increasing membrane curvature on the phase transition and mixing behaviour of a DMPC/DSPC lipid mixture as studied by Fourier transform infrared spectroscopy and differential scanning calorimetry. *Biophys. J.* 70:1373–1379.

Chapman, D. 1975. Phase-transitions and fluidity characteristics of lipids and cell membranes. *Q. Rev. Biophys.* 8:185–235.

Chervaux, C., and I. B. Holland. 1996. Random and directed mutagenesis to elucidate the functional importance of helix II and F-984 in the C-terminal secretion signal of *Escherichia coli* hemolysin. *J. Bacteriol.* 178:1232–1236.

Coote, J. G. 1992. Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiol. Rev.* 88:137–162.

de Kruijff, B. 1994. Anionic phospholipids and protein translocation. *FEBS Lett.* 346:78–82.

Dibble, A. R. G., M. D. Yeager, and G. W. Feigenson. 1993. Partitioning of gramicidin A′ between coexisting fluid and gel phospholipid phases. *Biochim. Biophys. Acta.* 1153:155–162.

Hannavy, K., S. Rospert, and G. Schatz. 1993. Protein import into mitochondria: a paradigm for the translocation of polypeptides across membranes. *Curr. Opin. Cell. Biol.* 5:694–700.

Isenman, L., C. Liebow, and S. Rothman. 1995. Transport of proteins across membranes. A paradigm in transition. *Biochim. Biophys. Acta.* 1241:341–370.

Jain, M. K., and D. Zakim. 1987. The spontaneous incorporation of proteins into preformed bilayers. *Biochim. Biophys. Acta.* 906:33–68.

Jorgensen, K., M. M. Sperotto, O. G. Mouritsen, J. H. Ipsen, and M. J. Zuckermann. 1993. Phase equilibria and local structure in binary lipid bilayers. *Biochim. Biophys. Acta.* 1152:135–145.

Knoll, W., K. Ibel, and E. Sackmann. 1981. Small angle neutron scattering study of lipid phase diagrams by the contrast variation method. *Biochemistry.* 20:6379–6383.

Lemmon, M. A., and D. M. Engelman. 1994. Specificity and promiscuity in membrane helix interactions. *FEBS Lett.* 346:17–20.

Levine, Y. K., and M. H. F. Wilkins. 1971. Structure of oriented lipid bilayers. *Nature New Biol.* 230:69–71.

Ludwig, A., R. Benz, and W. Goebel. 1993. Oligomerization of *Escherichia coli* hemolysin (HlyA) is involved in pore formation. *Mol. Gen. Genet.* 241:89–96.

Mateo, C. R., A. U. Acuña, and J. C. Brochon. 1995. Liquid-crystalline phases of cholesterol/lipid bilayers as revealed by the fluorescence of *trans*-parinaric acid. *Biophys. J.* 68:978–987.

Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* 858: 161–168.

McIntosh, T. J., R. V. McDaniel, and J. A. Simon. 1983. Induction of an interdigitated gel phase in fully hydrated phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 731:97–108.

McMullen, T. P. N., and R. N. McElhaney. 1995. New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochim. Biophys. Acta.* 1234:90–98.

Menestrina, G., C. Moser, S. Pellet, and R. Welch. 1994. Pore formation by *Escherichia coli* hemolysin (HlyA) and other members of the RTX toxins family. *Toxicology.* 87:249–267.

Menestrina, G., M. Ropele, M. Dalla Serra, C. Pederzolli, F. Hugo, S. Pellet, and R. A. Welch. 1995. Binding of antibodies to functional epitopes on the pore formed by *Escherichia coli* hemolysin in cells and model membranes. *Biochim. Biophys. Acta.* 1238:72–80.

Monette, M., M. R. Van Calsteren, and M. Lafleur. 1993. Effect of cholesterol on the polymorphism of dipalmitoylphosphatidylcholine/melittin complexes: an NMR study. *Biochim. Biophys. Acta.* 1149:319–328.

Nezil, F. A., and M. Bloom. 1992. Combined influence of cholesterol and synthetic amphiphilic peptides upon bilayer thickness in model membranes. *Biophys. J.* 61:1176–1182.

Ostolaza, H., B. Bartolomé, I. Ortiz de Zárate, F. de la Cruz, and F. M. Goñi. 1993. Release of lipid vesicle contents by the bacterial protein toxin α-hemolysin. *Biochim. Biophys. Acta.* 1147:81–88.

Ostolaza, H., B. Bartolomé, J. L. Serra, F. de la Cruz, and F. M. Goñi. 1991. α-Hemolysin from *E. coli*. Purification and self- aggregation properties. *FEBS Lett.* 280:195–198.

Ostolaza, H., A. Soloaga, and F. M. Goñi. 1995. The binding of divalent cations to *Escherichia coli* α-hemolysin. *Eur. J. Biochem.* 228:39–44.

Ostolaza, H., and F. M. Goñi. 1995. Interaction of the bacterial protein toxin α-hemolysin with model membranes: protein binding does not always lead to lytic activity. *FEBS Lett.* 371:303–306.

Piknová, B., D. Marsh, and T. E. Thompson. 1996. Fluorescence quenching study of percolation and compartmentalization in two-phase lipid bilayers. *Biophys. J.* In press.

Polozov, I. V., A. I. Polozova, Y. G. Molotkovsky, G. M. Anantharamaiah, J. P. Segrest, and R. M. Epand. 1995. Amphipathic peptide effects on the lateral domain reorganization of lipid bilayers. *Biophys. J.* 68:455A.

Pott, T., and E. J. Dufourc. 1995. Action of melittin on the DPPC-cholesterol liquid-ordered phase: a solid state ^{2}H and ^{31}P-NMR study. *Biophys. J.* 68:965–977.

Rytömaa, M., and P. K. J. Kinnunen. 1995. Reversibility of the binding of cytochrome c to liposomes. Implications for lipid-protein interaction. *J. Biol. Chem.* 270:3197–3202.

Sankaram, M. B., D. Marsh, L. M. Gierash, and T. E. Thompson. 1994. Reorganization of lipid domain structure in membranes by a transmembrane peptide: an ESR spin label study on the effect of the *Escherichia coli* outer membrane protein A signal peptide on the fluid phase domain connectivity in binary mixtures of dimyristoyl phosphatidylcholine and distearoyl phosphatidylcholine. *Biophys. J.* 66:1959–1968.

Sankaram, M. B., D. Marsh, and T. E. Thompson. 1992. Determination of fluid and gel domain sizes in two-component, two-phase lipid bilayers. An electron spin resonance spin label study. *Biophys. J.* 63:340–349.

Sankaram, M. B., and T. E. Thompson. 1991. Cholesterol-induced fluid-phase immiscibility in membranes. *Proc. Natl. Acad. Sci. USA.* 88: 8686–8690.

Stanley, P., L. C. Packman, V. Koronakis, and C. Hughes. 1994. Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science.* 266:1992–1995.

Surewicz, W. K., and R. M. Epand. 1984. Role of peptide structure in lipid-peptide interactions: a fluorescence study of the binding of pentagastrin-related pentapeptides to phospholipid vesicles. *Biochemistry.* 23:6072–6077.

Tomita, R., M. Watanabe, and T. Yasuda. 1992. Influence of membrane fluidity on the assembly of *Staphylococcus aureus* α-toxin, a channel-forming protein, in liposome membrane. *J. Biol. Chem.* 267: 13391–13397.

Vaz, W. L. C., E. C. C. Melo, and T. E. Thompson. 1989. Translational diffusion and fluid domain connectivity in a two-component, two phase phospholipid bilayer. *Biophys. J.* 56:869–876.

Virtanen, J. A., M. Ruonala, M. Vauhkonen, and P. Somerharju. 1995. Lateral organization of liquid-crystalline cholesterol- dimyristoylphosphatidylcholine bilayers. Evidence for domains with hexagonal and centered rectangular cholesterol superlattices. *Biochemistry.* 34: 11568–11581.

Vist, M. R., and J. H. Davis. 1990. Phase-equilibria of cholesterol dipalmitoylphosphatidylcholine mixture. ^{2}H-Nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.

Zhang, Y. P., R. N. A. H. Lewis, R. S. Hodges, and R. N. McElhaney. 1995. Peptide models of helical hydrophobic transmembrane segments of membrane proteins. 2. Differential scanning calorimetric and FTIR spectroscopic studies of the interaction of Ac-K_2-$(LA)_{12}$-K_2-amide with phosphatidylcholine bilayers. *Biochemistry.* 34:2362–2371.